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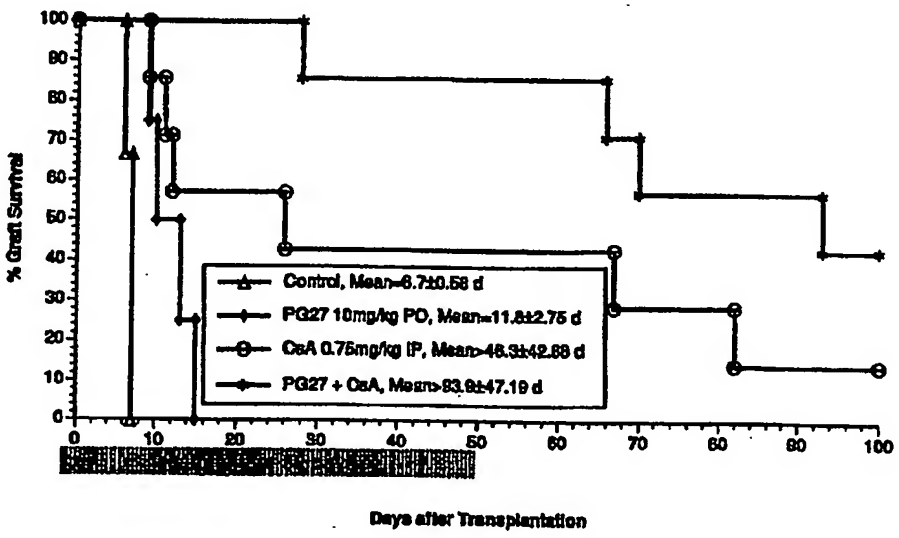

(57) Abstract

A composition for use in immunosuppression therapy is disclosed. The composition includes an immunosuppressant drug, such as cyclosporin A, in vehicle composed of an ethanol extract of the root xylem of Tripterygium wilfordii. The extract vehicle is effective alone, or in combination with such an immunosuppressant, in the treatment of transplantation rejection. Transplant survival time for untreated animals (open triangles), and animals treated with TW extract vehicle alone (solid diamonds), cyclosporin A alone (open circles), and cyclosporin A in a TW extract vehicle is disclosed.

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IMMUNOTHERAPY COMPOSITION AND METHOD

1. Field of the Invention

The present invention relates to a composition for use in immunotherapy, and to a composition for use in treating transplantation rejection.

2. References

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3. Background of the Invention

The immune system functions as the body's major defense against diseases caused by invading organisms. This complex system fights disease by killing invaders such as bacteria, viruses, parasites or cancerous cells while leaving the body's normal tissues unharmed. The immune system's ability to distinguish the body's normal tissues, or self, from foreign or cancerous tissue, or non-self, is an essential feature of normal immune system function. A second essential feature is memory, the ability to remember a particular foreign invader and to mount an enhanced defensive response when the previously encountered invader returns. The loss of recognition of a particular tissue as self and the subsequent immune response directed against that tissue produce serious illness.

An autoimmune disease results from the immune system attacking the body's own organs or tissues, producing a clinical condition associated with the destruction of that tissue. An autoimmune attack directed against the joint lining tissue results in rheumatoid arthritis; an attack against the conducting fibers of the nervous system results in multiple sclerosis. The autoimmune diseases most likely share a common pathogenesis and the need for safe and effective therapy.

Rheumatoid arthritis is one of the most common of the autoimmune diseases. Current treatments include three general classes of drugs (Schumacher, 1988): antiinflammatory agents (aspirin, non-steroidal antiinflammatory drugs and low dose corticosteroids); disease-modifying antirheumatic drugs, known as "DMARDs" (antimalarials, gold salts, penicillamine, and sulfasalazine) and immunosuppressive agents (azathioprine, chlorambucil, high dose corticosteroids,

cyclophosphamide, methotrexate, nitrogen mustard, 6-mercaptopurine, vincristine, hydroxyurea, and cyclosporin A). None of the available drugs are completely effective, and most are limited by severe toxicity.

Immunosuppressive agents are also used in treating or preventing transplantation rejection. Organ transplantation involving human organ donors and recipients (allogeneic grafts) and more recently, involving non-human primate donors and human recipients (xenogeneic grafts) has received considerable medical and scientific attention during the past two decades (Keown, 1991, Roberts, 1989, Platt, 1990). To a great extent, this effort has been aimed at eliminating, or at least reducing, the problem of rejection of the transplanted organ. In the absence of adequate immunosuppressive therapy, the transplanted organ is destroyed by the host immune system.

From follow-up studies on human transplant patients, as well as transplantation studies in animal model systems, the following features of transplant rejection have been established. The major targets in transplant rejection are non-self allelic forms of class I and class II major histocompatibility complex (MHC) antigens. Rejection is mediated by both antibodies and cytotoxic T lymphocytes (CTLs), with the participation of CD4+ "helper" T cells (Noelle, 1991). In general, foreign class I MHC antigens stimulate CD8+ CTLs, and foreign class II MHC antigens stimulate CD4+ T cells (Roitt, 1991).

Presently, the most commonly used agents for preventing transplant rejection include corticosteroids, cytotoxic drugs that specifically inhibit T cell activation such as azathioprine, immunosuppressive drugs such as cyclosporin A, and specific antibodies

directed against T lymphocytes or surface receptors that mediate their activation (Briggs, 1991). All of these drug therapies are limited in effectiveness, in part because the doses needed for effective treatment of transplant rejection may increase the patient's susceptibility to infection by a variety of opportunistic invaders, in part because of direct toxicity. Cyclosporin A, currently the most effective and most commonly used agent, is significantly toxic to the kidney. This nephrotoxicity limits the quantity of drug that can be safely given. The physician is frequently forced to administer sub-optimal doses of the drug because of this toxicity. A preparation that potentiates the action of cyclosporin A on the immune system, and thus allows the administration of lower doses of drug would be a considerable value in reducing the morbidity and mortality associated with transplantation.

4. Summary of the Invention

In one aspect, the invention includes a composition for use in immunosuppression therapy in a mammalian subject. The vehicle is composed of an ethanol extract from the root xylem of *Tripterygium wilfordii* and, carried in the vehicle, an immunosuppressant drug, particularly cyclosporin A, FK506, azathioprine, rapamycin, mycophenolic acid, or a glucocorticoid. The composition has an increased immunosuppressive activity with respect to a composition containing either the immunosuppressant drug or vehicle alone, allowing greater immunosuppression activity with reduced toxicity.

In one preferred embodiment, the composition of claim is used in treating transplantation rejection, and the immunosuppressant drug is cyclosporin A.

The vehicle may be a relatively unpurified ethanol extract, or may be purified to remove components which do not contribute to the potentiating effect of the vehicle. In one embodiment, the vehicle is composed of plant components which are (a) extractable from *T. wilfordii* root xylem by ethanol, (b) further extractable from ethanol:water (2:1) by methylene chloride; and (c) further retained on silica gel in 100% methylene chloride.

In a related embodiment, the vehicle is further refined to include plant components which are (a') further eluted from silica gel by methylene chloride:methanol 95:5; and (b') further contained in the intermediate fractions which are eluted from a silica gel column by elution with methylene chloride:methanol 97:3.

Also disclosed is the use of a plant-extract vehicle of the type for the manufacture of a medicament for use in treating transplantation rejection in a mammalian subject.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1 is a flow diagram of a method for preparing a *Tripterygium wilfordii* (TW) ethanol extract vehicle, in accordance with the invention, also showing additional purification methods that may be used to achieve more purified extract vehicles, for use in the composition of the invention;

Fig. 2A is a thin layer chromatogram of 1:1,000 TW extract vehicle (Lane A), 1:5,000 extract vehicle (Lane B) and purified 1:10,000 TW extract vehicle (Lane C);

Fig. 2B is a proton NMR spectrum of the purified TW extract vehicle;

Fig. 3 shows the suppressive effect of purified TW extract vehicle on production of IL-1b, TNF-alpha, IL-2, and IL-6 by human peripheral blood lymphocytes in culture;

Fig. 4 is a plot of transplant survival time for untreated animals (open triangles), and animals treated with TW extract vehicle alone (solid diamonds), cyclosporin A alone (open circles), and cyclosporin A in a TW extract vehicle; and

Fig. 5 is a plot of transplant survival time for untreated animals (squares), and animals treated with purified TW extract vehicle by oral administration (circles) or intraperitoneal administration (triangles).

Detailed Description of the Invention

I. Definitions

The terms below have the following meanings, unless specified otherwise:

"Immunosuppression therapy" refers to treatment of a mammalian subject, including a human subject, to suppress an autoimmune response or an immune response against a transplanted allogeneic or xenogeneic tissue or organ.

An "ethanol extract of the root xylem from *T. wilfordii*" refers to a composition containing ethanol-soluble components extracted from the root xylem of *T. wilfordii*; such a composition may include water soluble components that are also soluble in ethanol. The extract may be relatively unpurified, or may exist in progressively more purified form, as described below. For example, water-soluble components and components

which bind to silica gel in the presence of chloroform may be removed from the extract.

"Potentiated immunosuppression activity" refers to enhanced efficacy in immunosuppression therapy, as demonstrated by an enhanced therapeutic effect at a given immunosuppressant drug dose, or equivalent therapeutic effect at a reduced immunosuppressant drug dose.

II. *Tripterygium wilfordii* (TW) Extract Vehicle

A. Preparation of a TW Vehicle

The extract vehicle of the invention is obtained from the root xylem of *Tripterygium wilfordii* (TW), a medicinal plant which is grown in the Fujiang Province and other southern provinces of China. Plant material can be obtained easily in China. One method of preparation of a TW ethanol extract is illustrated in Fig. 1, and detailed in Example 1. Briefly, dried plant material is ground into a crude powder and then extracted by boiling in 95% ethanol with refluxing. The ethanol is removed and the extraction typically repeated twice. The resulting extracts are then combined and the ethanol removed (for example, by evaporation or heat-assisted evaporation. About 10 g dry ethanol extract is usually recovered per kg dry weight of plant material. This simple ethanol extract represents one preparation useful in the practice of the treatment method of the present invention. This vehicle is composed of plant components which are extractable from *T. wilfordii* root xylem by ethanol.

B. Methods for Further Purification of the TW Extract Vehicle

The ethanol extract vehicle from above may be further purified, to remove components, for example,

which do not contribute to the potentiation of immunosuppressant drug. An exemplary purification method is shown in Fig. 1, and detailed in Example 2. Briefly, the ethanol extract from above is filtered and volume is reduced under vacuum. The resulting syrup is diluted with water. Chloroform-soluble components are extracted by the adding chloroform, separating the non-aqueous-phase material, and discarding the aqueous-phase components. The chloroform extract can be concentrated, e.g., by evaporation, and applied to a silica gel column. The extract material is then eluted successively with chloroform and chloroform:methanol (95:5) and with chloroform:methanol (90:10), at a yield of about 1 g extract material per 20 g of original ethanol extract, corresponding to about 100 g dry weight starting plant material. This partial purified extract vehicle is referred to as a 1:1000 TW extract vehicle. The vehicle includes components which are further extractable from ethanol:water (2:1) by methylene chloride; further retained on silica gel in 100% methylene chloride; and further eluted from silica gel by methylene chloride:methanol 95:5.

The 1:1000 extract vehicle can be further purified (Example 2) by application to a silica gel column and elution with methylenechloride:methanol (97:3). Typically, six fractions are collected with the first and last of the six fractions to be discarded. The four intermediate eluted fractions are combined, with a yield of about 20 g material per 100 gram 1:1000 extract. The resulting extract is referred to herein as a 1:5000 extract vehicle.

The 1:5000) extract vehicle can be further purified by the same procedure, i.e., by elution on silica gel with methylene chloride:methanol (97:3) and

collection of the intermediate fractions, with a final yield of about 1 g material per 2 gram 1:5000 extract. This purified extract vehicle is referred to herein as a 1:10,000 extract vehicle, and includes components which are contained in the intermediate fractions which are eluted from a silica gel column by elution with methylene chloride:methanol 97:3.

It will be appreciated that the above purification steps are exemplary of the types of purification procedures that may be employed, if desired, to remove unneeded components from the ethanol-soluble TW extract vehicle. A variety of other methods chemical purification methods well known to those in the art may also be employed.

C. Physical Characteristics of Purified Vehicle

Fig. 2A shows a thin-layer chromatogram of the various extract TW extract vehicles prepared as above. Thin-layer chromatography and the chemical assays were carried out as described in Example 3. As seen from a comparison of the lanes:

(1) purification between the 1:1,000, 1:5,000 and 1:10,000 extract vehicles has removed a number of major plant components; and

(2) the final extract contains no alkaloids, as measured by application of the Dragendorff reagent.

Proton NMR (nuclear magnetic resonance), ^{13}C NMR were obtained using a 300 MHz General Electric QE Plus instrument. The proton NMR spectrum of the purified extract vehicle extract seen in Fig. 2B.

D. Biological Properties of Purified Vehicle

The 1:10,000 extract vehicle from above was examined for immunosuppressive activity in a variety of

biological assays. The following properties were observed:

1. The vehicle inhibited peripheral blood lymphocyte (PBL) proliferation that was stimulated by CD3 antibody. Increasing amounts of purified TW extract vehicle produced dose dependent inhibition, in the concentration range from about 0.3 to 1.25 μg/ml vehicle components/culture medium.

2. The effect of the purified TW extract vehicle on the production of the cytokines IL-1, TNF-alpha, IL-2, and IL-6 was assessed by measuring the concentration of these cytokines in the culture medium of anti-CD3 stimulated and unstimulated human PBLs. Cytokine levels were measured by standard ELISA methods using commercially available kits. Briefly, assay buffer was added to each of the wells of a microtiter plate containing pre-bound anti-cytokine antibody, followed by addition of standard or sample solution, diluted appropriately for the concentration range measured, followed by a second reporter-labeled antibody specific against the anti-cytokine antibody. Details are given in Example 4.

Figure 3 shows the levels of IL-1b, TNF-alpha, IL-2, and IL-6 in human PBLs at basal levels (solid bars), in cells stimulated with anti-CD3 (crosshatched bars), and in stimulated cells treated with purified TW extract vehicle (shaded bars). Anti CD3 stimulation resulted in a significant increase in all four cytokines measured. The purified TW extract significantly inhibited the production of TNF-alpha, IL-2, and IL-6, whereas the extract decreased production of IL-1b only slightly. Vehicle was added to the culture at a concentration of 5 μg/ml culture medium.

3. The ability of the extract to suppress the cell-proliferative effect of IL-1 in mouse thymocytes (O'Gara, 1990), was also examined. Almost complete inhibition of cell proliferation was observed in the range of 0.01 to 1 μg dried vehicle components/ml culture medium.

4. The vehicle also suppressed, but at higher concentration, the cell-proliferative activity of IL-2 on the IL-2 dependent cell line, HT-2, according to published methods (Watson, 1979).

5. Potential cytotoxicity of the vehicle was assessed by measuring the effect of the purified extract on the ability of cultured cells to reduce MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), an index of cellular respiration. This is a sensitive assay for the detection of cytotoxicity (Green, 1984). Details of the procedure are given in Example 5. In addition, staining with the vital dye trypan blue, which stains dead cells, was also carried out routinely in all of the culture systems described. Toxicity was evaluated *in vitro* in two different cell culture systems, human PBLs and mouse thymocytes. No vehicle cytotoxicity was observed at a vehicle concentration of 10 mg/ml in PBLs and 3.1 mg /ml in mouse thymocytes, the highest concentrations tested in each system.

6. A measure of *in vivo* immunosuppression is inhibition of cell proliferation in the mixed lymphocyte reaction (MLR) (Bradley; Mishell, 1980). In these experiments, mice were treated with the TW extract vehicle for 14 days. Spleen cells, the "responder" cells, were prepared and co-cultured with irradiated spleen cells prepared from a different mouse strain, the "stimulator" cells. The responder cells proliferate in the presence of the allogenic stimulator

cells. Irradiation of the stimulator cells renders them unable to proliferate. After a 72 hour incubation, tritiated thymidine was added to the mixed cell cultures, and incorporation of the labeled nucleotide into DNA was measured as an index of cell proliferation.

In the MLR test reported in Example 8, C3H mice were injected intraperitoneally with either 1 or 10 mg purified TW extract vehicle/Kg body weight. Animals were treated daily for 14 days prior to harvesting the spleen cells. Spleen cells from C3H mice were cultured with irradiated spleen cells from BalbC or C57 Black (C57Bl) mice. Irradiated spleen cells from C3H mice served as controls.

Irradiated allogeneic spleen cells stimulated C3H cell proliferation 2-4 fold, in comparison with irradiated syngeneic cells. The extract vehicle effectively inhibited the mixed lymphocyte response, and was dose dependent in the range 1-10 mg/Kg animal weight.

In a similar series of experiments, C3H mice were treated for 14 days with 10 mg purified TW extract vehicle/kg body weight or with diluent alone. Spleen cells were harvested and their response to the mitogens Phytohemagglutinin (PHA) and Concanavalin A (ConA) was assessed. A similar immunosuppressive effect by the vehicle was observed.

III. Immunosuppressant Composition

In one aspect, the invention includes a pharmaceutical composition which includes an immunosuppressant drug in a TW extract vehicle of the type described above. The immunosuppressant drug is one of the following:

(a) Cyclosporin A or cyclosporin C ("cyclosporin"), a non-polar cyclic oligopeptide;

(b) FK506, a fungal macrolide immunosuppressant;

(c) azathioprine, or 6[(1-Methyl-4-nitro-1H-immadazole-5yl)thio]1H-purine;

(d) rapamycin, a fungal macrolide immunosuppressant;

(e) mycophenolic acid, or 6-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxy-5-isobenzofuranyl)-4-methyl-4-hexanoic acid; and

(f) an immunosuppressant glucocorticoid, such as prednisone or dexamethasone.

The vehicle is a mixture of ethanol-soluble TW plant components, as described above. The proportions of the two components is preferably in the range 0.5 to 10 parts by weight immunosuppressant drug to 1-50 parts by dry weight vehicle components, where the immunosuppressant drug is dissolved or suspended in a liquid form of the vehicle, or mixed with a dried, powdered form of the vehicle.

The composition may be administered to a subject orally, transdermally or parenterally, e.g., intravenous, subcutaneous, intraperitoneal, or intramuscular injection.

When the composition is employed in the form of solid preparations for oral administration, the preparations may be tablets, granules, powders, capsules or the like. In a tablet formulation, the composition extract is typically formulated with additives, for example, an excipient such as a saccharide or cellulose preparation, a binder such as starch paste or methyl cellulose, a filler, a disintegrator and so on, all being ones usually used in the manufacture of medical preparations.

For use in oral liquid preparation, the composition may be prepared as a liquid suspension, emulsion, or syrup, being supplied either in liquid form or a dried form suitable for hydration in water or normal saline.

The compositions of the present invention may be injected in the form of aqueous solutions, suspensions or oily or aqueous emulsions, such as liposome suspensions. Typically, for parenteral administration, the composition is formulated as a lipid, e.g., triglyceride, or phospholipid suspension, with the extract vehicle components including a liquid carrier, and the immunosuppressant being dissolved or suspended in the lipid phase of the suspension.

IV. Treatment Method

The composition is employed in immunosuppression therapy, in particular, therapy in treating autoimmune disease or transplantation rejection.

Table 1 below gives a list of autoimmune diseases which are appropriate for immunotherapy.

Table 1

Autoimmune Diseases	
Disease	Tissue Affected
Addison's disease	adrenal
Allergies	inflammatory cells
Asthma	bronchi
Atherosclerosis	vessel walls
Crohn's disease	intestine
Diabetes (Type I)	pancreas
Graves' disease	thyroid
Guillain-Barré Syndrome	nerve cells
Systemic Lupus erythematosis (SLE)	multiple tissues
Multiple sclerosis	nerve cells
Myasthenia Gravis	neuromuscular junction
Psoriasis	skin
Primary biliary cirrhosis	liver
Rheumatoid arthritis	joint lining
Uveitis	eye

In treatment an autoimmune condition, the patient is given the compound on a periodic basis, e.g., 1-2 times per week at a dosage level sufficient to reduce symptoms and improve patient comfort.

For treating rheumatoid arthritis, the composition may be administered by intravenous injection or by direct injection into the affected joint. The patient may be treated at repeated intervals of at least 24 hours, over a several week period following the onset of symptoms of the disease in the patient.

For the treatment of systemic lupus erythematosis (SLE), as another example, the composition may be administered by oral or parenteral administration, such as IV administration. The dose that is administered is preferably 25-75% of the dose of the immunosuppressant drug that would be administered, when

given in the absence of the *Tripterygium wilfordii* extract vehicle. The amount of vehicle administered is preferably in the range 1 to 25 mg/Kg patient body weight per day, with lower amounts being preferred for parenteral administration, and higher amounts being preferred for oral administration. Parenteral administration may be by injection, e.g., intravenously, intramuscularly, or subcutaneously, inhalation, or uptake via a mucosal membrane.

For therapy in transplantation rejection, the method is intended particularly for the treatment of rejection of heart, kidney, liver, and bone marrow transplants. The method may also be used in the treatment of graft-versus-host disease, in which transplanted immune cells attack the allogeneic host. Initial treatment is administered perioperatively. In addition, the composition may be administered chronically to prevent graft rejection, or in treating acute episodes of late graft rejection. As above, the dose administered is preferably 25-75% of the normal dose of the immunosuppressant drug alone, where the amount of vehicle administered is in the range 1-25 mg dried extract material/Kg body weight. The dose may be increased or decreased appropriately, depending on the response of the patient, and over the period of treatment, the ability of the patient to resist infection. According to an important feature of the invention, the effective dose of immunosuppressant drug is reduced substantially by its formulation in the TW extract vehicle, allowing higher drug doses to be administered and/or more prolonged treatment without serious side effects.

The treatment is typically started soon after the surgical transplantation procedure, and is continued on a daily dosing regimen, for a period of at least

several weeks, for treatment of acute transplantation rejection. "During the treatment period, the patient may be tested periodically for immunosuppression level, e.g., by a mixed lymphocyte reaction involving allogenic lymphocytes, or by biopsying the transplanted tissue.

The treatment of transplantation rejection, in accordance with the invention is illustrated by the heart transplantation model reported in Example 6. The method involves a well-characterized rat model system (Ono and Lindsey, 1969) in which a transplanted heart is attached to the abdominal great vessels of the recipient animal, and the viability of the transplanted heart is gauged by the heart's ability to beat in the recipient animal.

The animals were treated from one day preceding to 52 days following heart transplantation with (i) control solution (5% ethanol, 10ml/Kg), (ii) purified extract vehicle (oral administration, 10 mg/Kg), (iii) cyclosporin A (intraperitoneal (IP) administration, 0.75 mg/Kg), or (iv) cyclosporin A (0.75 mg/Kg) in purified extract vehicle (10 mg/Kg), administered IP.

As seen in Fig. 4, the group treated with ethanol vehicle alone had a mean graft survival time of 6.7 days. In the group treated with the 10 mg/Kg TW extract vehicle, mean graft survival increased to 11.8 days. With IP administration of cyclosporin A, mean graft survival time increased to 46.3 days, and there was a small percentage of grafts surviving at 100 days. The most effective results were observed in treatment with the composition of the invention, where mean graft survival time was 93.5 days, and nearly half of the grafts survived to 100 days.

V. Transplantation Rejection Treatment with Vehicle Alone

In another aspect, the invention includes treating transplantation rejection by administration to a subject of the TW extract vehicle alone. The vehicle is administered orally, or parenterally, in the doses given above, and employing the dosing schedules given above.

In Fig. 5 is plotted the effect of the treatment method on allograft survival. The three groups are (a) 5% alcohol solution (squares), (b) 10 mg/Kg purified TW extract administered daily by oral administration (circles), and (c) 10 mg/Kg purified TW extract administered daily by intraperitoneal injection (triangles). There were three animals in each group. Cardiac allograft survival was measured by the presence of detectable graft heart beat.

Graft survival times are shown in Fig. 5. As seen, the hearts grafts were rejected by the untreated recipient animals in 6.7 days. In the animals treated with purified TW extract by oral administration, the allografts remained viable for 17 days, 3 days following discontinuation of treatment. In the animals treated with purified TW extract by intraperitoneal injection, the allografts remained viable for 21.3 days, one week after discontinuation of treatment. Details of the treatment method are given in Example 10. There was not evidence of toxicity in the treated animals.

From the foregoing, it can be appreciated how various objects and features of the invention are met. The purified extract material is readily prepared in quantity from root xylem of *Tripterygium wilfordii*, in a final amount which is about 0.1% of the original ethanol extract. The material can be administered

orally and is effective in prolonging transplant survival in mammals, as shown by the treatment method in Example 8. At the same time, the purified extract appears to have low toxicity, allowing long-term treatment with fewer side effects.

The following examples illustrate the method for obtaining purified extract, and demonstrate various physical, chemical, *in vitro*, and *in vivo* properties of the extract. The examples are intended to illustrate, but in no way limit the scope of the invention.

Example 1

Preparing *Tripterygium wilfordii* Ethanol Extract Vehicle

Tripterygium wilfordii plants were obtained in Fujiang Province, China. Plants were air dried in sunlight. The root xylem of the plants (300 g) was ground into a crude powder and extracted with 5 volumes (1.5 l) of 95% ethanol, under reflux at 85°C for 4 hours. The filtered xylem powder was then extracted two more times in 3 volumes (900 ml total) of 95% ethanol. The three extracts (total of about 3,3, l) were combined and the resulting mixture was concentrated at 50°C under vacuum, to about 2% of the original volume, i.e., about 66 ml.

Example 2

Further Purifications of the TW Extract Vehicle

A. The CH_2Cl_2 TW Extract Vehicle

The ethanol extract syrup obtained in Example 1 was then diluted with 33 ml water, filtered through Whatman # 1 filter paper. The filtrate was extracted 4 times (50 ml/extraction) with methylene chloride (CH_2Cl_2).

B. The 1:1,000 TW Extract Vehicle

The combined, CH_2Cl_2-extract filtrate (about 200 ml) was concentrated, and applied to a 1 cm (diameter) × 5 cm column containing silica gel (1.5 kg; 60-200 mesh). The column was washed successively with 100 ml methylene chloride, and 100 ml methylene chloride:methanol (95:5). The fraction which eluted in 95:5 solvent contained about 0.3 g material, and is referred to herein as a 1:1000 extract vehicle.

C. The 1:5,000 TW Extract

Forty grams of 1:1,000 vehicle prepared as described above (in scale-up) was concentrated to a small volume in 20 ml acetone. The solution was applied to a 13 cm × 14 cm column containing silica gel (800 gm; 60-200 mesh) and eluted with methylene chloride:methanol 97:3 to produce six 1 liter ml fractions. The yield of each fraction was about 5% or 2 grams. Fractions 2-5 were combined and the resulting 8 grams of material are referred to herein as the 1:5000 TW extract vehicle.

D. The 1:10,000 TW Extract Vehicle

Fractions 2-5 were combined and the resulting 8 grams of material, the 1:5000 TW extract vehicle, was then applied to a 8 cm × 40 cm column containing silica gel (320 gm; 260-400 mesh) and eluted with methylene chloride:methanol (97:3) to produce five 300 ml fractions. Fractions 2-4, which were yellowish in color, were combined. The solvent was removed by evaporation under vacuum to yield 4 grams of light brown powder, referred to herein as the purified (1: 10,000) TW extract.

Example 3

Thin-Layer Chromatography of Purified TX Vehicle

One microgram samples of extracts were applied to a silica gel coated aluminum thin layer chromatography plate (Whatman, catalog # 4420 222). The development solvent was hexane:methylene chloride:methanol in volume ratios of 1:1:0.15. Following separation, samples were visualized using an ultraviolet lamp and by application of an aerosol of 0.5% vanillin in H_2SO_4-ethanol (4:1).

TLC profiles of the various TW extracts are shown in Figure 2. Lane A shows the 1:1,000 extract, lane B shows the 1:5,000 extract and lane C the 1:10,000 extract, herein called the purified TW extract. It can be seen that purification between the 1:1000, 1:5000 and 1:10000 extract vehicles has removed a number of major plant components. For these TW extracts, the thin layer chromatographic profile showed no alkaloid in the extract, as determined by application of the Dragendorff reagent.

The thin layer chromatographic profile showed no alkaloid in the extract, as determined by application of the Dragendorff reagent.

Example 4

Effect of Purified TW Vehicle on Cytokine Production

The ability of the purified *T. wilfordii* extract to affect the production of IL-1, TNF-alpha, IL-2, and IL-6, cytokines secreted by anti CD3 stimulated (X-35 antibody, 5 ng/ml) and unstimulated human PBLs, was measured.

PBLs were prepared, incubated and treated as described above. The purified TW extract was used at 5 μg/ml. Samples of tissue culture medium were col-

lected at the end of 24 hours incubation and stored at -70°C prior to assay.

Cytokine measurements were carried out using commercially available ELISA assay kits (R&D Systems), in accordance with the supplier's protocols. In brief, 100 μl of the assay buffer supplied was added to each of the wells of a microtiter plate containing pre-bound anti-cytokine antibody, followed by 100 μl of standard or sample solution, diluted appropriately for the concentration range measured. All incubations were carried out at 37° or 24°C, per the supplier's protocol. Following two hours incubation, the plates were washed four times with assay buffer, and the second antibody, anti-cytokine-horseradish peroxidase (HRP), was added to each well in a volume of 200 μl. Following a second 2 hour incubation, the wells were washed four times with buffer, and 200 μl of HRP substrate was added to the wells. After 20 minutes incubation, the reaction was terminated by addition of 50 μl H_2SO_4 to each well. Optical density was determined using a Molecular Devices microtiter plate reader.

As shown in Figure 4, basal levels of IL-1, TNFa, IL-2 and IL-6 increased markedly (3.8 to 167.3 pg/ml, 30.9 to 654.8 pg/ml, 7.6 to 148.7 pg/ml, and 108.5 to 2646 pg/ml, respectively) with X-35 stimulation. At a concentration of 5 μg/ml, the purified TW extract inhibited this X-35 stimulated increase by 16, 89, 93 and 100%, respectively. The extract most likely inhibits cytokine production, though the decrease in medium cytokine concentration could theoretically result from increased catabolism. Decreased cytokine production may be responsible, at least in part, for the decrease in PBL proliferation

in vitro and for the immunosuppressive effect of the extract *in vivo*.

Example 5

Cytotoxicity of Extract Vehicle

Potential cytotoxicity of the purified extract was assessed by measurement of the extract's effect on the ability of cultured cells to reduce MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). MTT, a yellow-colored compound, is reduced by mitochondrial enzymes to form a purple crystalline reduction product (formazan), providing an index of cellular respiration as well as a sensitive assay for cytotoxicity (Green).

Cytotoxicity was assessed in both cultured PBLs and thymocytes (Green). A stock solution of MTT (Sigma Chemical Co., St. Louis, MO), 5 mg MTT/ml phosphate buffered saline, pH 7.4, was prepared and stored in the dark at 4°C. Following 21 hours incubation under conditions identical to those used in the assays, 25 μl of MTT solution was added to each culture well. After an additional 3 hour incubation, the experiment was terminated by addition of a solution of 10% sodium dodecyl sulfate in 0.01 N HCl. Following overnight incubation at 37°C (to solubilize the purple crystals, the MTT reduction product), optical density was determined at 570-650 nm in a Molecular Devices microtiter plate reader. Data are expressed as the ratio of the optical density of the extract treated sample to that of untreated controls.

Example 6

Treatment of Heart Transplant Rejection

A. Transplantation Method

Heterotopic whole heart transplantation was performed according to the standard method (Ono). The donor (Brown Norway (200-255g) rats, Charles River, Wilmington, MA) and the recipient (Adult male Lewis rats, 225-275g, Charles River) were anesthetized with sodium pentobarbital (40 mg/kg). After the donor was heparinized, the heart graft was removed and stored at 4°C in PhysioSol Irrigation Solution (Abbott Laboratories, N. Chicago, IL). The ascending aorta and pulmonary artery were transsected, and the vena cava and pulmonary veins were ligated. The recipient abdominal aorta and inferior vena cava were exposed through a median abdominal incision. The donor heart aorta and pulmonary artery were anastomosed end-to-side to recipient's infrarenal abdominal aorta and inferior vena cava, respectively, with running 8-0 monofilament nylon suture (Ethilon, Inc., Somerville, NJ). Because of the functional properties of the aortic valve, blood did not enter the left ventricle but rather flowed through the coronary arteries to the right atrium, pulmonary artery and the recipient vena cava. The cold ischemic time of all the cardiac grafts was less than 45 minutes. Graft heartbeat was monitored by abdominal palpation. The period of functional graft survival was measured as the number of days during which cardiac graft contractions could be detected by abdominal palpation. Results were confirmed by direct visualization at laparotomy.

B. Treatment with Cyclosporin Composition

Rats (3 animals/group) were treated for a total of 52 days with (i) control solution (5% ethanol,

10ml/Kg), (ii) purified extract vehicle (oral administration, 10 mg/Kg), (iii) cyclosporin A (intraperitoneal (IP) administration, 0.75 mg/Kg), or (iv) cyclosporin A (0.75 mg/Kg) in purified extract vehicle (10 mg/Kg), administered IP.

The treatment methods started on the day prior to surgery and continuing daily until postoperative day 52, or until the end of allograft survival. Each graft recipient was followed until the graft ceased beating. The results are seen in Fig. 4, and are discussed above.

C. Treatment with TW Extract Vehicle

The transplantation model described in Part A was employed. The animals (3 animals/group) were treated for 14 days with the extract vehicle (10mg/kg) or with ethanol solution (5% ethanol, 10ml/kg), starting on the day prior to surgery and then daily. The extract vehicle was administered both orally and by intraperitoneal injection. Each graft recipient was followed until the graft ceased beating. The results are shown in Fig. 5, described above.

Although the invention has been described with respect to particular methods and applications, it will be appreciated that various changes and modifications may be made without departing from the spirit of the invention.

IT IS CLAIMED:

1. A composition for use in immunosuppression therapy in a mammalian subject, comprising

a vehicle composed of an ethanol extract from the root xylem of *Tripterygium wilfordii*, and

carried in the vehicle, an immunosuppressant drug selected from the group consisting of cyclosporin A, FK506, azathioprine, rapamycin, mycophenolic acid, and a glucocorticoid,

said composition having a potentiated immunosuppression activity with respect to a composition containing either the immunosuppressant drug or vehicle alone.

2. The composition of claim 2, for use in treating transplantation rejection in the subject.

3. The composition of claim 2, wherein the immunosuppressant drug is cyclosporin A.

4. The composition of claim 1, wherein the vehicle is composed of plant components which are:

(a) extractable from *T. wilfordii* root xylem by ethanol;

(b) further extractable from ethanol:water (2:1) by methylene chloride; and

(c) further retained on silica gel in 100% methylene chloride.

5. The composition of claim 1, wherein the vehicle is composed of plant components which are:

(a') further eluted from silica gel by methylene chloride:methanol 95:5; and

(b') further contained in the intermediate fractions which are eluted from a silica gel column by elution with methylene chloride:methanol 97:3.

6. The composition of claim 1, 9, wherein the vehicle is substantially free of alkaloids.

7. The use of an ethanol extract from the root xylem of *Tripterygium wilfordii* for the manufacture of a medicant for use in treating transplantation rejection in a mammalian subject.

8. The use of claim 7, wherein the vehicle is composed of components which are:

(a) extractable from *T. wilfordii* root xylem by ethanol;

(b) further extractable from ethanol:water (2:1) by methylene chloride; and

(c) further retained on silica gel in 100% methylene chloride.

9. The use of claim 8, wherein the vehicle is composed of plant components which are:

(a') further eluted from silica gel by methylene chloride:methanol 95:5; and

(b') further contained in the intermediate fractions which are eluted from a silica gel column by elution with methylene chloride:methanol 97:3.
wherein the human.

10. The use of claim 7, wherein the vehicle is substantially free of alkaloids.

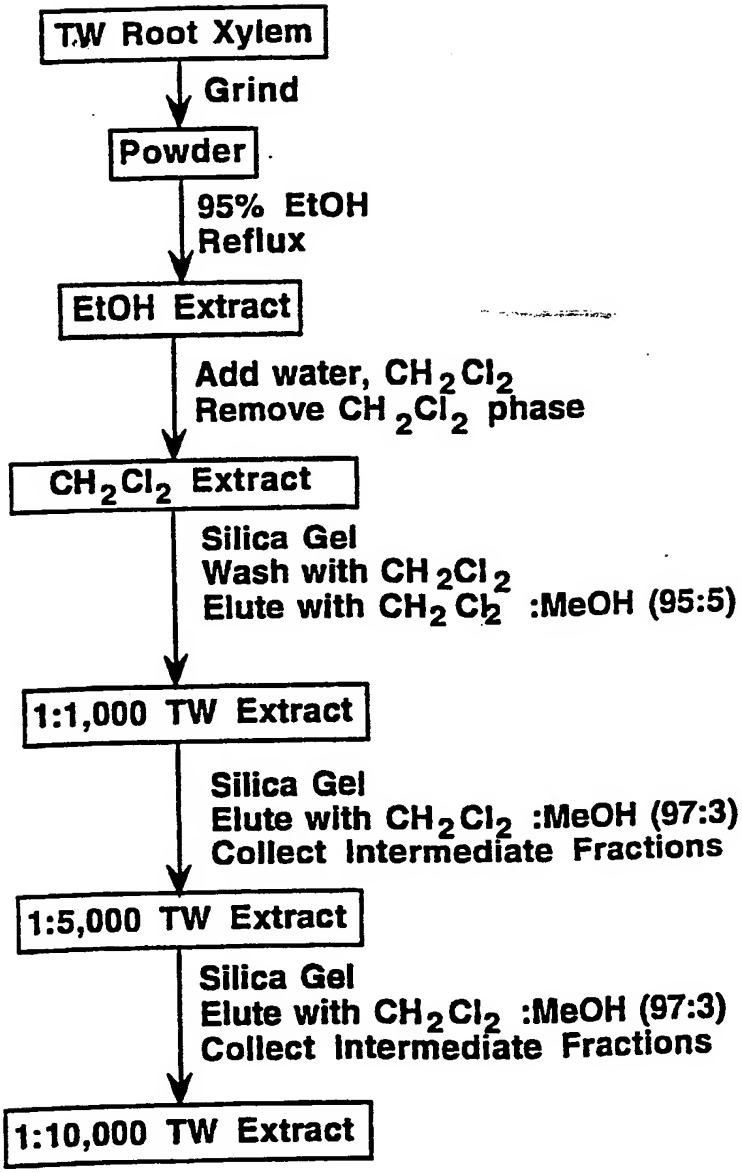
TW Root Xylem
Grind
Powder
95% EtOH
Reflux
EtOH Extract
Add water, CH_2Cl_2
Remove CH_2Cl_2 phase
CH_2Cl_2 Extract
Silica Gel
Wash with CH_2Cl_2
Elute with CH_2Cl_2 :MeOH (95:5)
1:1,000 TW Extract
Silica Gel
Elute with CH_2Cl_2 :MeOH (97:3)
Collect Intermediate Fractions
1:5,000 TW Extract
Silica Gel
Elute with CH_2Cl_2 :MeOH (97:3)
Collect Intermediate Fractions
1:10,000 TW Extract

Fig. 1

Fig. 2A

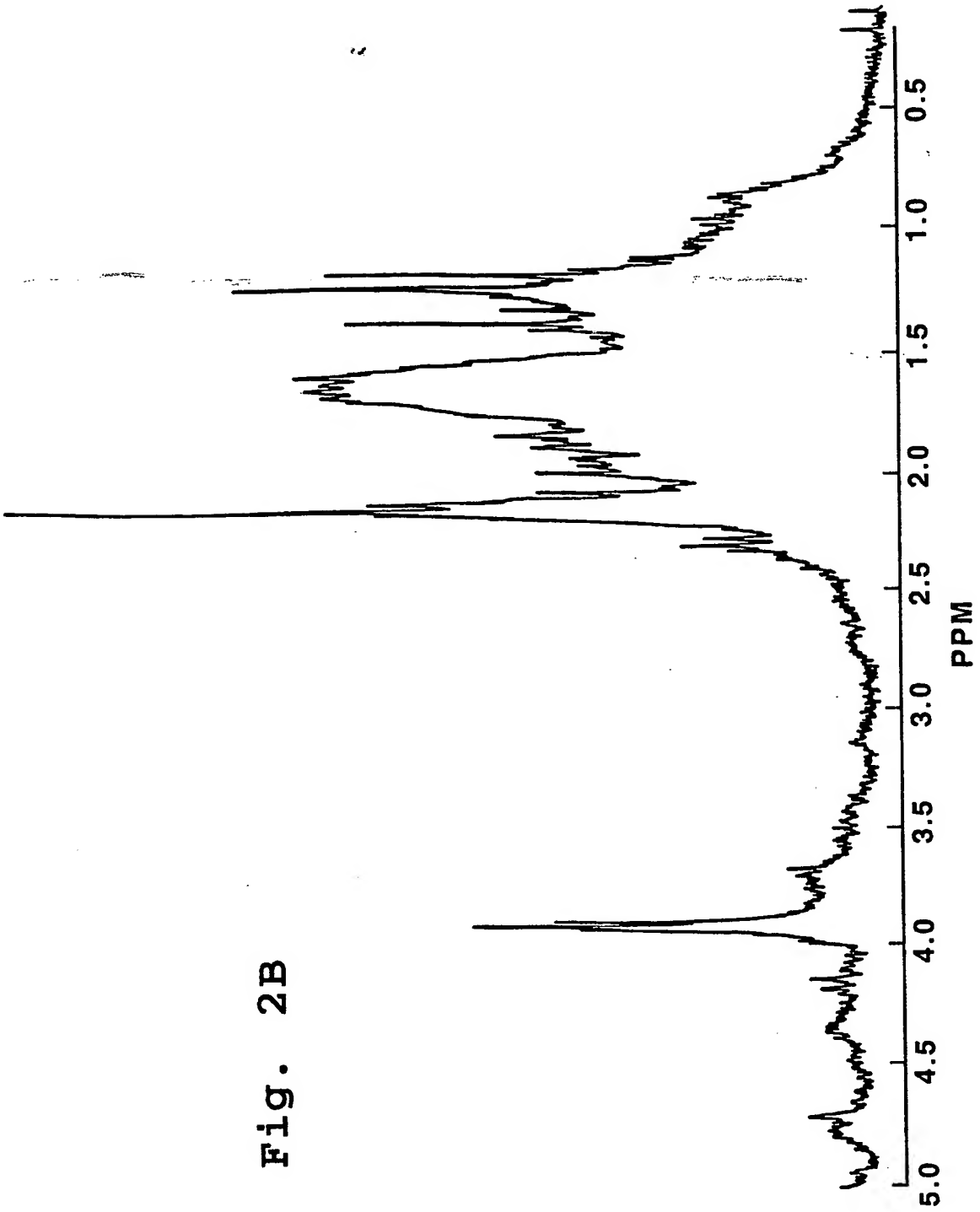

Fig. 2B

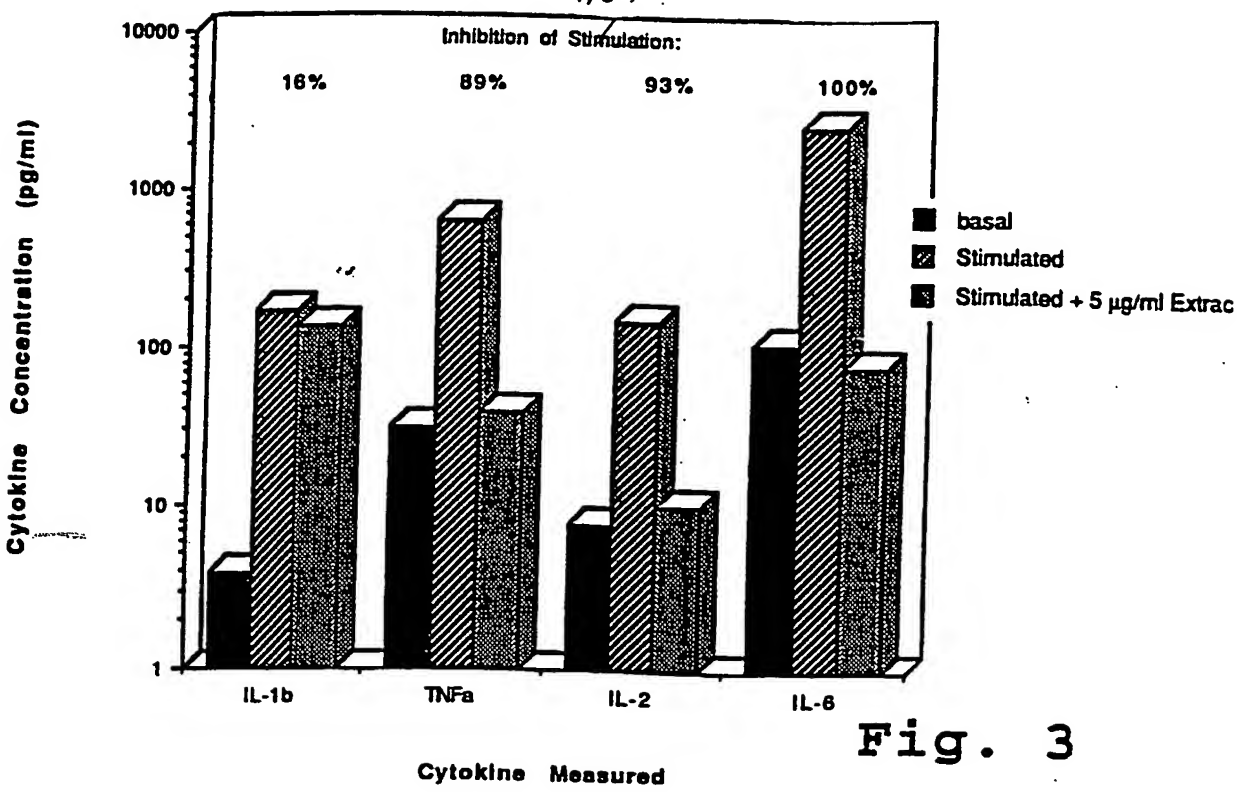
Inhibition of Stimulation:
16%
89%
93%
100%
Cytokine Concentration (pg/ml)
10000
1000
100
10
1
basal
Stimulated
Stimulated + 5 µg/ml Extrac
IL-1b
TNFa
IL-2
IL-6
Cytokine Measured

Fig. 3

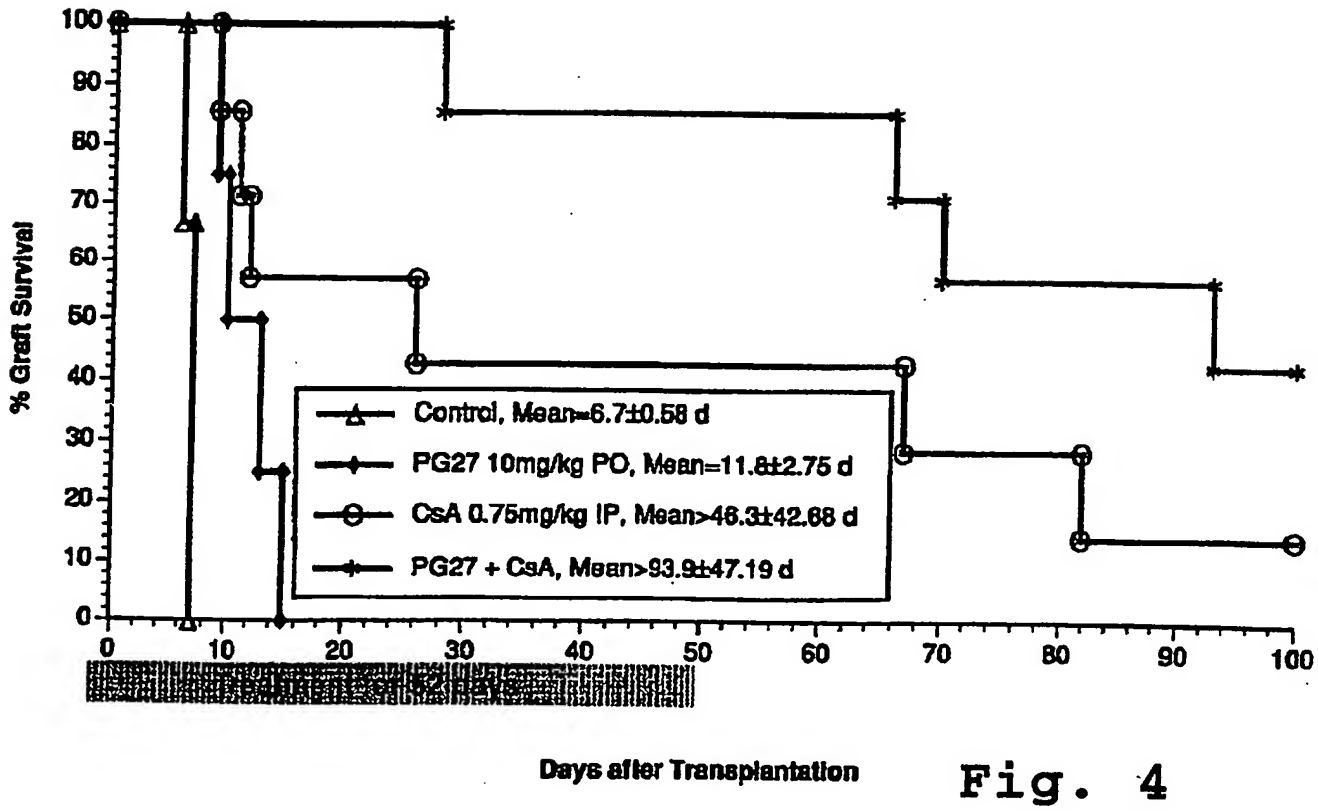
% Graft Survival
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
Control, Mean=6.7±0.58 d
PG27 10mg/kg PO, Mean=11.8±2.75 d
CsA 0.75mg/kg IP, Mean>46.3±42.68 d
PG27 + CsA, Mean>93.9±47.19 d
Days after Transplantation

Fig. 4

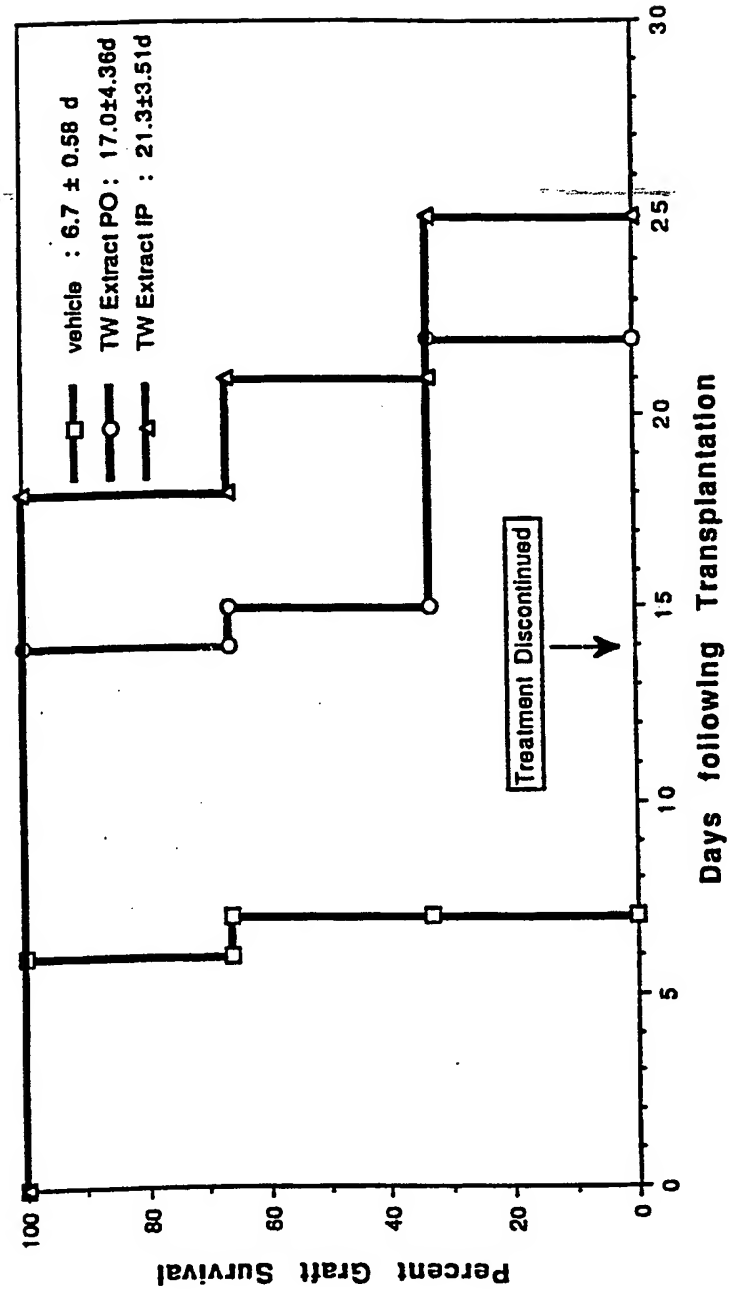
vehicle : 6.7 ± 0.58 d
TW Extract PO : 17.0±4.36d
TW Extract IP : 21.3±3.51d
Treatment Discontinued
Percent Graft Survival
Days following Transplantation
0
20
40
60
80
100
0
5
10
15
20
25
30

Fig. 5

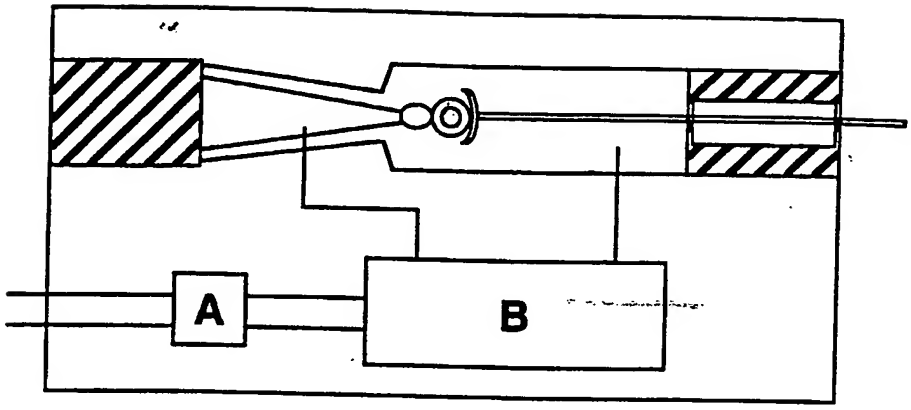

Fig. 21

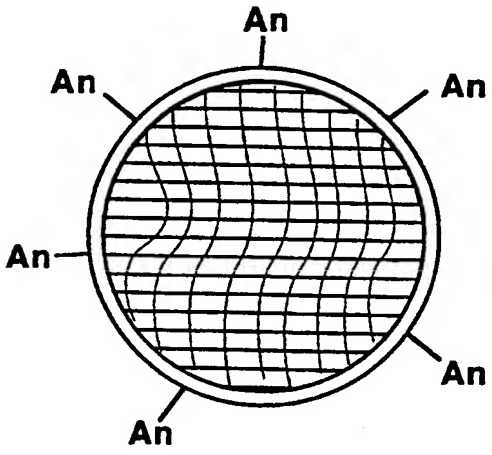

Fig. 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10724

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 35/78, 37/00, 31/66, 31/56, 31/43, 31/52, 31/36
US CL :424/195.1; 514/9, 116, 179, 198, 265, 465, 885
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/195.1; 514/9, 116, 179, 198, 265, 465, 885

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,005,108 (Kupchan et al) 25 June 1977, Abstract, col. 2, lines 3-68- col. 3, lines 1-8.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 January 1994	Date of mailing of the international search report JAN 26 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JOHN W. ROLLINS Telephone No. (703) 308-0196

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